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DIAGNOSTIC AND TREATMENT METHODS RELATING TO AUTOSOMAL DOMINANT NOCTURNAL FRONTAL LOBE EPILEPSY (ADNFLE)

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(57)

The present invention provides a method for diagnosing autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). This method comprises: obtaining a sample of body fluid or tissue from a subject suspected of suffering from ADNFLE; isolating chromosomal material (DNA) from that sample; sequencing the CHRNA4 gene of chromosome 20q13.2-q13.3, at least in the region of exon 5; comparing that sequence to a reference sequence relating to the normal CHRNA4 gene; and thereby determining whether said subject has an ADNFLE-inducing mutation of the CHRNA4 gene. As an alternative to comparison with the normal sequence, the sample may be compared to a reference sequence relating to the specific abnormality suspected to be present. Mutations of interest are those which have the effect of changing the structure of one of the subunits of the neuronal nicotinic acetylcholine receptor (nAChR), more especially the ion channel thereof. Of particular interest are mutations at codon 248, which have the effect of replacing serine by another amino acid (eg phenylalanine) in the sixth amino acid of the transmembrane domain 2 (M2) of the α4 subunit of nAChR.

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ORIGINAL

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Invention title: "Diagnostic And Treatment Methods Relating To Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE)"

Details of Associated Provisional Application No: PN 3840 dated 28th June 1995.

The following statement is a full description of this invention, including the best method of performing it known to us.

BACKGROUND TO THE INVENTION

Epilepsy is one of the most common brain disorders. In its various clinical forms, it affects at least 2% of the population, at some time in their lives. Although epilepsy has long been thought to have a genetic basis, it has proven difficult to identify the relevant genes.

Our research has, for the first time, identified the genetic cause of a form of partial epilepsy. Our research has led to the provision of diagnostic methods for Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE), and also has implications with respect to pharmaceutical intervention.

The epilepsies comprise a group of syndromes, that are divided into generalised and partial (focal) types. Familial occurrence has long been recognised, but progress in mapping epilepsy genes has been slow, except for rare cases where the inheritance is easily determined from classical genetic studies.

Our research centred on a large South Australian family, spanning six generations, with 27 members affected by ADNFLE.

The disorder first manifests itself in early childhood, and persists throughout adult life. It is a form of partial epilepsy, causing frequent, violent brief seizures at night. ADNFLE is frequently misdiagnosed as nightmares, other sleep disorders, hysteria or non-epileptic movement disorders. Because the problem always occurs at night, the disorder can be debilitating.

Penetrance is 75%, and there is considerable variation in the severity amongst affected family members. Severely affected family members rarely, if ever, have an uninterrupted night's sleep, and do not wake refreshed.

By studying the pedigree of this South Australian family, we were able to assign the gene associated with ADNFLE to chromosome 20q13.2-q13.3. The relevant coding region is exon 5 of the CHRNA4 gene on that chromosome.

The CHRNA4 gene encodes the α4 subunit of the neuronal nicotinic acetylcholine receptor (nAChR). A missense mutation in exon 5 of the CHRNA4 gene results in the complex aromatic amino acid phenylalanine being replaced by the neutral serine in the 6th amino acid position of the transmembrane domain 2 (M2) of the α4 subunit of nAChR. This mutation has been found to be associated with ADNFLE.

SUMMARY OF THE INVENTION

The present invention provides a method for diagnosing ADNFLE. This method comprises: obtaining a sample of body fluid or tissue from a subject suspected of suffering from ADNFLE; isolating chromosomal material (DNA) from that sample; sequencing the CHRNA4 gene of chromosome 20q13.2-q13.3, at least in the region of exon 5; comparing that sequence to a reference sequence relating to the normal CHRNA4 gene; and thereby determining whether said subject has an ADNFLE-inducing mutation of the CHRNA4 gene. As an alternative to comparison with the normal sequence, the sample may be compared to a reference sequence relating to the specific abnormality suspected to be present. Mutations of interest are those which have the effect of changing the structure of one of the subunits of the neuronal nicotinic acetylcholine receptor (nAChR), more especially the ion channel thereof. Of particular interest are mutations at codon 248, which have the effect of replacing serine by another amino acid (eg phenylalanine) in the sixth amino acid of the transmembrane domain 2 (M2) of the α4 subunit of nAChR. However, there are other possible mutations that would lead to amino acid substitutions in transmembrane domain 2 or other parts of the gene which could cause epilepsy.

The amino acid sequence of the α4 subunit of normal nAChR, and the DNA sequence coding therefor, are shown in Figures 1A and 1B respectively. The particular mutation identified in our study of a family affected by ADNFLE occurs at codon 248 in a region coding for the ion channel of subunit α4 of nAChR. The normal TCC codon, encoding serine, is replaced by TTC, encoding phenylalanine.

Purified and isolated DNA molecules of particular interest are outlined in accompanying claims 1 to 6.

This discovery of the α4 subunit of the nicotinic acetylcholine receptor (nAChR) as a defective gene in this form of epilepsy suggests new avenues for pharmacological treatment of epilepsy and the development of new anti-epileptic drugs. The acetylcholine system has not previously been implicated in human epilepsy. This finding strongly suggests that agents that augment this system may be effective anti-epileptic agents in this and related epilepsies. Moreover, there is experimental evidence that anti-cholinesterase inhibitors such as physostigmine act as allosteric modulators of the nicotinic receptor, in addition to increasing acetylcholine availability. Thus, this class of drugs may represent a hitherto unknown group of effective anti-epileptic agents. Now, specific pharmacological agents can be developed to correct the known defect in this and related epilepsies.

Accordingly, further aspects of the present invention are as follows:-

(a) A method of treating or preventing epilepsy, in particular ADNFLE, by administration of a pharmaceutical agent which has the effect of augmenting the acetylcholine system, eg by improving the function of the ion channel of the α4 subunit of nAChR; and

(b) A pharmaceutical agent for the prophylaxis or treatment of epilepsy, in particular ADNFLE, comprising a compound which has the effect of augmenting the acetylcholine system, eg by improving the function of the ion channel of the

α4 subunit of nAChR, together with conventional adjuvants and pharmaceutical vehicles.

DETAILED DESCRIPTION OF THE INVENTION

The gene for ADNFLE was assigned to chromosome 20q13.2-q13.3 in one large Australian kindred with 27 affected individuals (1). The neuronal nicotinic acetylcholine receptor α4 subunit (CHRNA4) maps to the same region of 20q (2). Its gene is expressed in all layers of the frontal cortex (3). CHRNA4 consists of six exons spanning approximately 10 kb of genomic DNA. Exon 5 contains the main coding region, including the agonist binding sites (4) and three of the four transmembrane domains. As a first step towards identification of the gene responsible for ADNFLE, genomic DNA from affected family members was screened for mutations within the CHRNA4 gene. To this end we designed primers for amplification of the coding regions directly from genomic DNA. It turned out that all the affected members of the ADNFLE family, together with three obligate carriers and two non-penetrant individuals, possessed a Ser248Phe mutation in the α4 subunit.

The main coding region distributed in exon 5 of the CHRNA4 gene was amplified from the genomic DNA of ADNFLE patients belonging to the previously described chromosome 20 linked family (1). Altered mobility in single strand conformation analysis (SSCA) was detected for two overlapping fragments (5). The fragments were subcloned and sequenced after amplification with vector specific primers (6). Furthermore, direct sequencing of PCR fragments from genomic DNA was performed. The clones carrying the aberrant allele showed a C to T transition (Figure 2). The missense mutation replaces the neutral serine by the complex aromatic phenylalanine in the sixth amino acid position (homologous to Ser248 of the Torpedo α subunit; 7) of the transmembrane domain 2 (M2). Database analysis showed that serine is conserved in this position in most of the other subunits of the human neuronal nicotinic acetylcholine receptor (nAChR). Only in subunits α7 and α8, which diverged very early in the history of the multigene family of nAChR subunits and were found to have distinct structural and pharmacological properties, serine is replaced by another amino acid

residue (8). Furthermore, Ser248 is found in species evolutionarily as diverse as the goldfish *Carassius auratus* and the locust *Schistocerca gregaria* (Figure 3).

A PCR assay was developed, with one of the primers carrying a one base mismatch near the 3'-end, creating a new HpaII restriction site in the wild-type but not in the mutant allele (9). This allowed us to rapidly search for mutant alleles in all family members and in a control population. Furthermore, the nature of the mutation was confirmed by an independent test. The mutation was not present in two different control samples comprising a total of 333 individuals (232 randomly collected German blood donors and 101 unrelated Australian individuals).

The mutant allele was found to cosegregate with the disease (Figure 4). All the clinically affected family members available for investigation carried the mutation. The unaffected family members tested, including nine spouses marrying into the family, were homozygous for the wild-type allele. The non-penetrant individuals III-1, III-3 and III-10, who are obligatory carriers of the disease gene, possessed the mutant allele. Individual VI-10 was unaffected at the age of 3 years. As the median age of onset of ADNFLE is 8 years, it is uncertain if he is pre-symptomatic or a non-penetrant carrier. The unaffected individuals IV-4 and V-42 who are not obligatory carriers also proved to possess the mutant allele. This can be explained, again, by incomplete penetrance. The segregation of D2OS19 alleles through this family gave a lod score of 9.29 at a recombination frequency of zero (1), which in the absence of flanking markers leads to a carrier risk of 94% or greater for IV-4, V-42 and VI-10. This was determined on the basis of a 90% support interval for the recombination frequency by a method as previously described (10). The M2 variant of CHRNA4 can be regarded as a genetic marker segregating in this family. The lod score between this marker and ADNFLE was 11.17 at a recombination frequency of zero.

Neuronal nicotinic acetylcholine receptors (nAChRs) consist of heterologous pentamers comprising various combinations of approximately 10 subunits (α2- α8; β2- β4) which are differentially expressed throughout the brain to form physiologically and pharmacologically distinct receptors. In chicken and rat, the predominant nAChR

subtype is composed of α4 and β2 subunits (11-13). The M2 segments of the subunits are arranged as α-helices and contribute to the walls of the neurotransmitter-gated ion channel. The α-helices appear to be kinked and orientated in such a way that the side chains of the highly conserved M2-leucine residues project inwards when the channel is closed. ACh is thought to cause a conformational change by altering the association of the amino acid residues of M2. The opening of the channel seems to be due to rotations of the gate forming side chains of the amino acid residues; the conserved polar serines and threonines may form the critical gate in the open channel (14-17).

The missense mutation at Ser248 of the M2 segment of the CHRNA4 gene observed in this family with ADNFLE is unlikely to be an innocent polymorphism for a number of reasons. First, it is strongly linked to the ADNFLE locus and was not found in a large number of controls. Second, the mutated serine is highly conserved, not only among different human subunits of the nicotinic acetylcholine receptor, but also in the corresponding subunits of other vertebrates and some nonvertebrates (Figure 3). Third, Ser248 residues from the five subunits of nAChR appear to constitute a critical narrow ring in the transmembrane ion channel, and form the binding site of noncompetitive inhibitors including chlorpromazine and phencyclidine (14,17). Fourth, experimental mutations of Ser248 to valine or tyrosine decrease sodium and potassium conductance, and substitution of alanine alters the dissociation rate of channel blockers (14). The mutation in this family, replacing serine by the neutral complex aromatic phenylalanine, might similarly be expected to have a deleterious effect on the properties of the open channel.

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5. 2.5 µl of the PCR products were mixed with 4µl of 95% formamide, 20 mM EDTA, 0.05% Xylene xyanol, 0.05% bromphenol blue. After heat denaturation, the fragments were separated overnight on a 10% polyacrylamide gel at room temperature or at 4°C and silver stained. PCR products showing a band shift were subcloned and analysed under the same SSCA (single strand conformation analysis) conditions to identify the clones carrying the mutant allele prior to sequencing. Sequencing was performed either as double strand sequencing of miniprep DNA or as single strand sequencing of PCR products (which had been amplified with one of the primers biotinylated at the 5'-end) according to the Dynabeads-Streptavidin protocol (Dynal) using Sequenase version 2.0 (USB).

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9. Part of exon 5 was amplified using the following primers: A,5'GGCGAGTGGGTCATCGTGG 3' and B,5'GGTGAGCGACAGCAGCCCG 3' with 32 cycles and an annealing temperature of 65°C. The mismatching third base on the 3' end of primer B was introduced to create an artificial HpaII restriction site in the wild-type but not in

the mutant allele. 5 µl of the PCR products were digested to completion with HpaII in a total volume of 15 µl and analyzed on a 10% polyacrylamide gel. The following band pattern was observed: wild-type allele: 26 bp, 72 bp, 122 bp; mutant allele: 26 bp, 72 bp, 122 bp, 141 bp.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A purified and isolated DNA molecule comprising all or part of the CHRNA4 gene of chromosome 20q13.2-q13.3, said molecule encoding a subunit of the neuronal nicotinic acetylcholine receptor (nAChR).

2. A purified and isolated DNA molecule comprising all or part of the CHRNA4 gene of chromosome 20q13.2-q13.3, said molecule encoding part or all of the ion channel of nAChR.

3. A purified and isolated DNA molecule comprising all or part of the CHRNA4 gene of chromosome 20q13.2-q13.3, said molecule encoding the transmembrane domain 2 of the α4 subunit of nAChR.

4. A purified and isolated DNA molecule comprising exon 5 of the CHRNA4 gene of chromosome 20q13.2-q13.3.

5. A purified and isolated DNA molecule according to any one of claims 1 to 4, said molecule comprising part or all of the DNA sequence shown in Fig 1.

6. A purified and isolated DNA molecule according to any one of claims 1 to 4, said molecule comprising part or all of the DNA sequence shown in Fig 1, but with replacement of the normal codon TCC at position 248 with the codon TTC.

7. A method for diagnosing autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) comprising:

(a) obtaining a sample of body fluid or tissue from a subject suspected of suffering from ADNFLE;

(b) isolating chromosomal material (DNA) from the sample obtained in step (a);

(c) sequencing the CHRNA4 gene of chromosome 20q13.2-q13.3 of the chromosomal material isolated in step (b), at least in the region of exon 5; and

(d) comparing that sequence to a reference sequence relating to either a normal or an abnormal CHRNA4 gene, to thereby determine whether said subject has an ADNFLE-inducing mutation of the CHRNA4 gene.

8. A method according to claim 7, wherein the ADNFLE-inducing mutation has the effect of changing the structure of one of the subunits of the encoded neuronal nicotinic acetylcholine receptor (nAChR).

9. A method according to claim 7 or claim 8, wherein the ADNFLE-inducing mutation has the effect of changing the structure of the ion channel of the encoded nAChR.

10. A method according to any one of claims 7 to 9, wherein the ADNFLE-inducing mutation leads to an amino acid substitution in the transmembrane domain 2 of the α4 subunit of the encoded nAChR.

11. A method according to any one of claims 7 to 10, wherein the ADNFLE-inducing mutation is in exon 5 of the CHRNA4 gene.

12. A method according to any one of claims 7 to 11, wherein the ADNFLE-inducing mutation is at codon 248 of the CHRNA4 gene.

13. A method according to claim 12, wherein the ADNFLE-inducing mutation is replacement of the normal codon (TCC) by TTC.

14. A method according to any one of claims 7 to 13, wherein the reference sequence of step (d) is all or part of the DNA sequence encoding the α4 subunit of normal nAChR, as shown in Fig 1.

15. A method according to any one of claims 7 to 14, substantially as described herein.

16. A method of treating or preventing ADNFLE by administration of a pharmaceutical agent which has the effect of augmenting the acetylcholine system.

17. A method according to claim 16, wherein the pharmaceutical agent has the effect of improving the function of the ion channel of the α4 subunit of nAChR.

18. A pharmaceutical agent for the prophylaxis or treatment of ADNFLE comprising a compound which has the effect of augmenting the acetylcholine system together with conventional adjuvants and pharmaceutical vehicles.

19. A pharmaceutical agent according to claim 18, wherein the pharmaceutical agent has the effect of improving the function of the ion channel of the α4 subunit of nAChR.

Dated this 28th day of June, 1996.

WOMEN'S AND CHILDREN'S HOSPITAL,
UNIVERSITY OF BONN and
UNIVERSITY OF MELBOURNE
By their Patent Attorneys
MADDERNS

ABSTRACT

The present invention provides a method for diagnosing autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). This method comprises: obtaining a sample of body fluid or tissue from a subject suspected of suffering from ADNFLE; isolating chromosomal material (DNA) from that sample; sequencing the CHRNA4 gene of chromosome 20q13.2-q13.3, at least in the region of exon 5; comparing that sequence to a reference sequence relating to the normal CHRNA4 gene; and thereby determining whether said subject has an ADNFLE-inducing mutation of the CHRNA4 gene. As an alternative to comparison with the normal sequence, the sample may be compared to a reference sequence relating to the specific abnormality suspected to be present. Mutations of interest are those which have the effect of changing the structure of one of the subunits of the neuronal nicotinic acetylcholine receptor (nAChR), more especially the ion channel thereof. Of particular interest are mutations at codon 248, which have the effect of replacing serine by another amino acid (eg phenylalanine) in the sixth amino acid of the transmembrane domain 2 (M2) of the α4 subunit of nAChR.

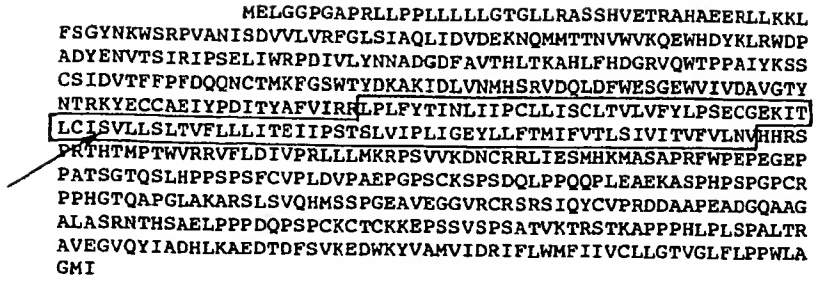

Fig. 1A The amino acid sequence of the α4 subunit of normal nACh R as deposited in the GENBANK database. The amino acid sequence of the region containing the ion channel is boxed. The serine mutated in ADNFLE is arrowed.

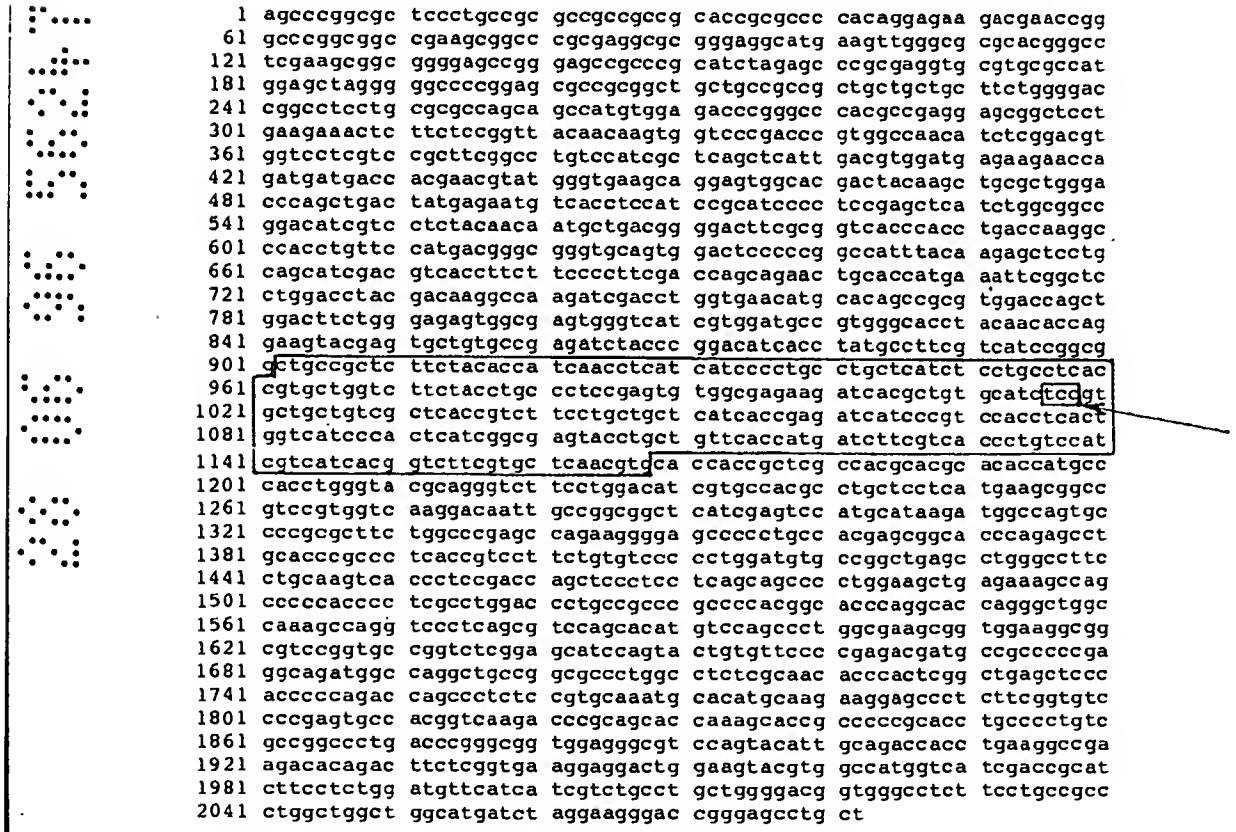

Fig. 1B The DNA sequence of the α4 subunit of normal nAchR as deposited in the GENBANK database. The DNA sequence of the region containing the ion channel is boxed. Codon 248 (serine codon) is arrowed.

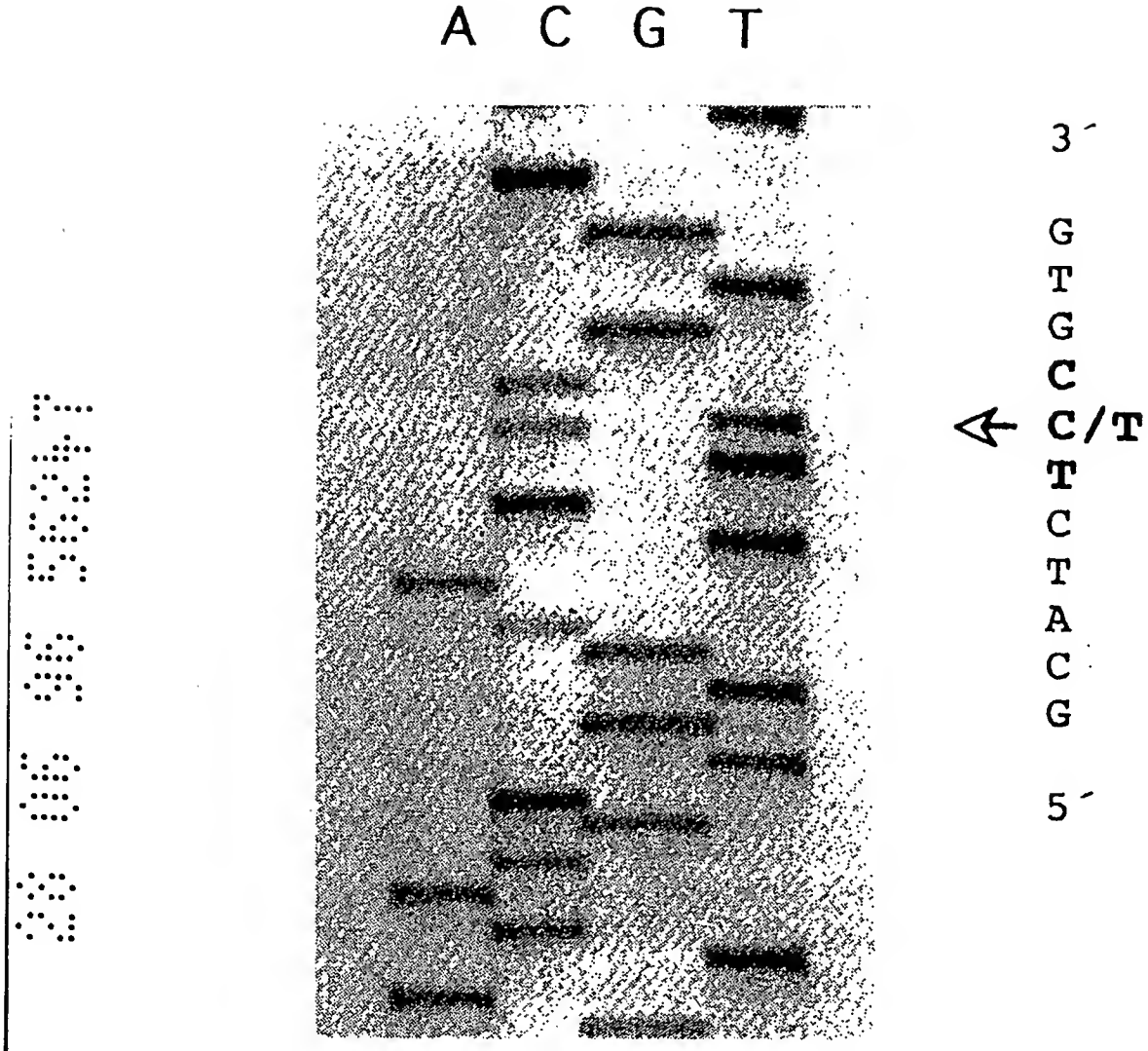

Fig. 2. Direct sequencing of the missense mutation in an ADNFLE patient. Genomic DNA was amplified with one 5´ biotinylated and one non- biotinylated exon 5 primer. Single strands were isolated using the Dynabeads-M-280 streptavidin system (Dynal) and sequenced with an internal primer. The position of the amino acid transition (the normal and the mutated nucleotide are present due to the sequencing of both alleles) is indicated at the right.

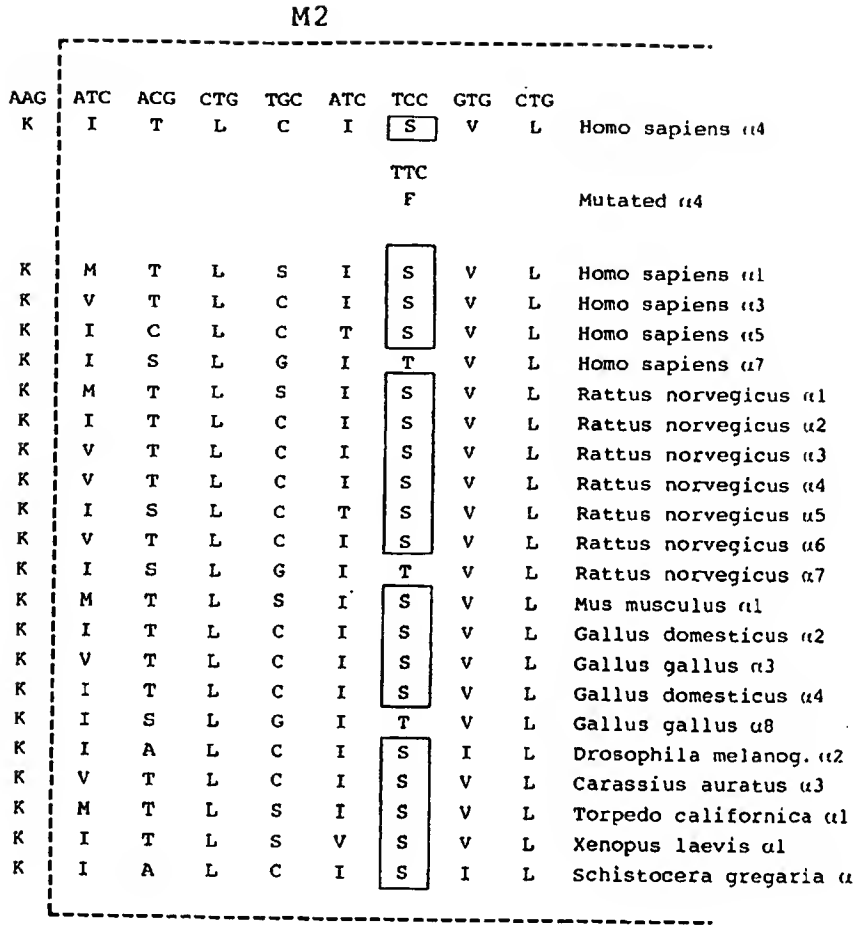

Fig.3 Amino-acid homologies in the second transmembrane domain. Parts of the nucleotide and amino acid sequence from transmembrane domain 2 of the human *CHRNA4* gene including the missense mutation are shown, compare with amino acid sequences from other human α subunits and with subunits from different species (obtained from the EMBL database). The position of Ser 248 is indicated by boxes.

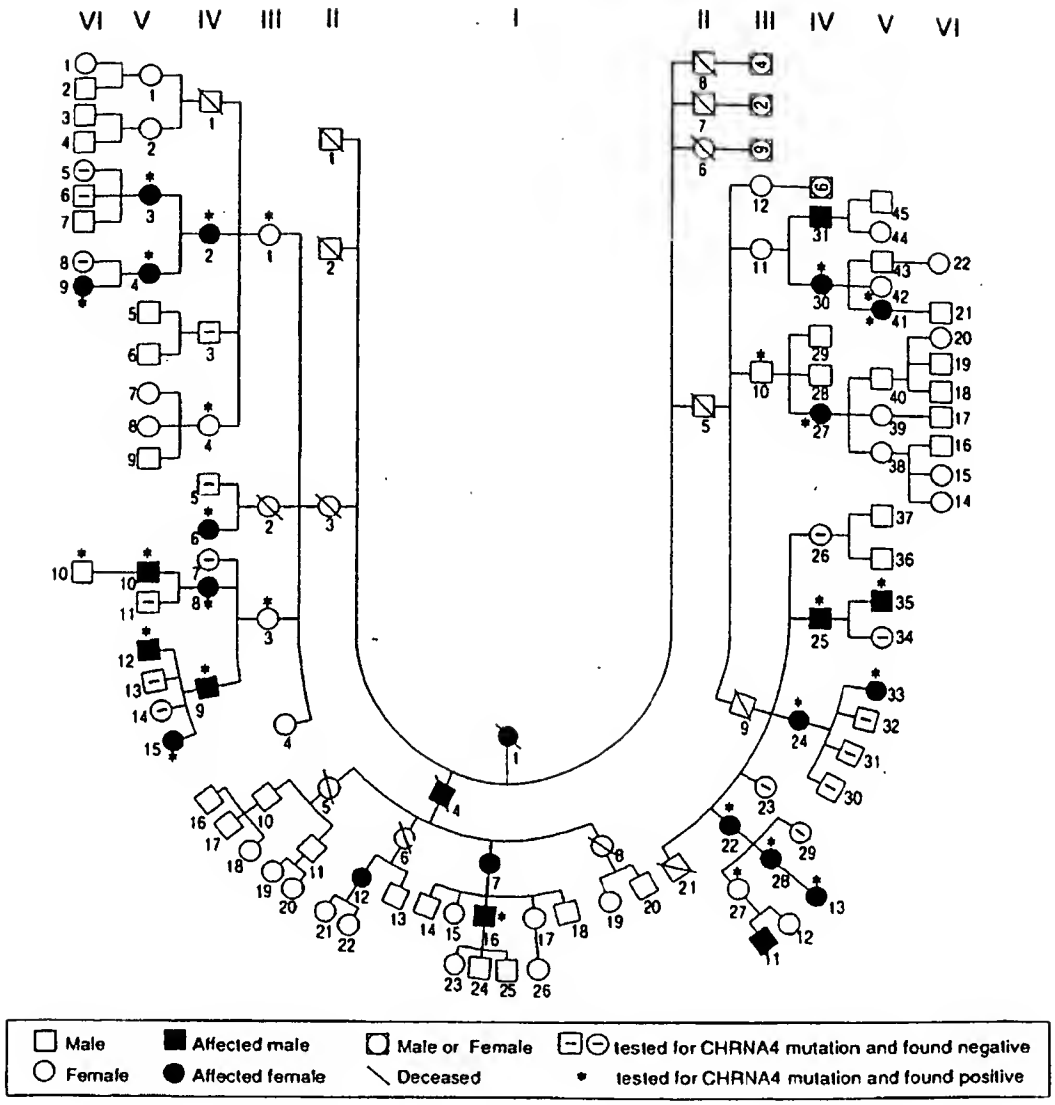

Fig. 4 Family with autosomal dominant nocturnal frontal lobe epilepsy showing individual status for the *CHRNA4* mutation. Nine spouses who married into the pedigree and were negative for the mutation are not shown.